Oecologia (2017) 184:739–748
DOI 10.1007/s00442-017-3882-8

HIGHLIGHTED STUDENT RESEARCH

Evaluating local adaptation of a complex phenotype: reciprocal tests of pigmy rattlesnake venoms on treefrog prey

Sarah A. Smiley-Walters[1,2] · Terence M. Farrell[2] · H. Lisle Gibbs[1]

Received: 23 August 2016 / Accepted: 8 May 2017 / Published online: 17 May 2017

Abstract Theory predicts that predator–prey interactions can generate reciprocal selection pressures on species pairs, which can result in local adaptation, yet the presence and pattern of local adaptation is poorly studied in vertebrate predator–prey systems. Here, we used a reciprocal common garden (laboratory) experimental design involving comparisons between local and foreign populations to determine if local adaptation was present between a generalist predator—the pigmy rattlesnake (*Sistrurus miliarius*)—and a co-occurring prey—the squirrel treefrog (*Hyla squirella*). We conducted toxicity trials using snake venom from two populations separated by 340 km tested on prey from sympatric and allopatric populations, resulting in data from four venom origin–frog origin combinations. We assessed venom effectiveness using two measures (frog mortality at 24 h and time to frog death) and then used regression analyses to look for a signal of local adaptation with either measure. We found evidence for local adaptation for one measure (time to death), but not the other (frog mortality). We argue that in this system, the time to death of a prey item is a more ecologically relevant measure of venom effectiveness than is frog mortality at 24 h. Our results document an example of local adaptation between two interacting vertebrates using a whole-organism assay and a local versus foreign criteria and provide evidence that population-level variation in snake venom is adaptive.

Keywords Predator–prey interactions · Fitness trade-off · Common garden · Snake venom · *Sistrurus* · Toxicity

Communicated by Lin Schwarzkopf.

We document local adaptation of a complex trait in a predator–prey interaction between two vertebrates. Our study demonstrates that population-level variation in snake venom has adaptive significance.

✉ Sarah A. Smiley-Walters
smiley-walters.1@osu.edu

[1] Department of Evolution, Ecology and Organismal Biology, The Ohio State University, Columbus, OH 43210, USA

[2] Department of Biology, Stetson University, Deland, FL 32723, USA

Introduction

Antagonistic interactions between species, such as those between predator and prey, can create strong selection pressures that are important for shaping evolutionary trajectories (Hanifin et al. 2008). Moreover, the nature of species interactions, especially in species with broad geographic ranges, may not be static across all populations because of spatial variation in abiotic and biotic factors (Benkman et al. 2001; Callaway et al. 2002). This selection landscape creates divergent pressures, which can result in local adaptation as populations evolve traits that enable them to have higher fitness in local compared to foreign environments (reviewed by Kawecki and Ebert 2004). Understanding local adaptation is important because it has broad implications in ecology, evolution, and conservation, including potential roles in the process of speciation (Schemske 2010; Lenormand 2012), coevolutionary dynamics including host-parasite and predator–prey systems (Greischar and Koskella 2007; Hoeksema and Forde 2008; Keogh et al. 2016; Holding et al. 2016), and the ability of species to adapt to changing climates (St Clair and Howe 2007; Schiffers et al. 2012; Golbuu et al. 2016; Mathiasen and Premoli 2016; Mosca et al. 2016).

In practice, there are several definitions used to identify the pattern of local adaptation, including home versus away, local versus foreign, and sympatric-allopatric contrasts (Kawecki and Ebert 2004; Blanquart et al. 2013). When applied to populations within a species, the home versus away criterion compares the fitness of a population in two environments; local adaptation is present when a population has higher fitness in its home environment than in geographically distinct 'away' environments (Kawecki and Ebert 2004). The local versus foreign definition compares two populations within an environment and is based on the concept that local adaptation is present when a population has higher fitness in its' local environment than in any foreign population (Kawecki and Ebert 2004). Lastly, the sympatric-allopatric contrast is a broader definition of local adaptation that focuses on the difference between the average fitness in all sympatric population-environment combinations and the average fitness in allopatric population-environment combinations; populations must have higher fitness in sympatric combinations for local adaptation to be present (Kawecki and Ebert 2004; Blanquart et al. 2013). Debate remains concerning which of these theoretical definitions is the best to use in practice—the local versus foreign criterion is the more rigorous definition advocated by Kawecki and Ebert (2004), while the sympatric-allopatric contrast is favored by Blanquart et al. (2013) because of its greater power in detecting local adaptation. Note that these criteria for defining local adaptation are not independent of one another (Kawecki and Ebert 2004). The local versus foreign definition of local adaptation is graphically visible as a classic interaction pattern in plots of fitness across environments (Kawecki and Ebert 2004; Brennan et al. 2016). Finally, while testing for local adaptation by any criteria is ideally done with multiple populations, the significance of the sympatric-allopatric contrast cannot be evaluated with only two populations (Kawecki and Ebert 2004; Blanquart et al. 2013).

Any definition of local adaptation requires measurement of a trait in which variation has clear functional consequences. Snake venom is one such trait because it has a direct effect on the ability of an individual snake to immobilize (Zimmerman et al. 1990; Richards et al. 2012; Torres-Bonilla et al. 2016) and digest (Thomas and Pough 1979) its prey. In this study, we examine interactions between a venomous snake and its prey. The composition of snake venom is a complex phenotypic trait with a strong genetic basis (Wooldridge et al. 2001; Li et al. 2005; Casewell et al. 2013; Dagda et al. 2013). Venom is comprised of a mixture of enzymes, non-enzymatic proteins, and other compounds that cause physical damage and disruption of homeostasis in the envenomated prey (reviewed by Mackessy 2008), allowing prey to be immobilized prior to ingestion. Pit viper venoms may also facilitate prey digestion (Thomas and Pough 1979; but see Chu et al. 2009) as well as enhance chemosensory location of envenomated prey following snake strike and release behaviors (Chiszar et al. 1999). In addition to predation, venom may also play a role in snake defense (Hayes et al. 2002; Jansa and Voss 2011; Voss and Jansa 2012).

Snake venom is variable across multiple biological scales (individual to family-level) (reviewed by Chippaux et al. 1991), yet the evolutionary significance of this variation is unclear. Two competing hypotheses exist for the processes that generate and maintain variation in snake venom—the first is that variation evolves via neutral processes such as genetic drift (Williams et al. 1988) while the second is that variation in venom is adaptive, driven by selection acting through diet variation, enabling snakes in different geographical locations to better subdue specific prey (Daltry et al. 1996). Recent evidence for a role of natural selection in shaping venom variation has come from the demonstration of prey-specific effects of venom (Barlow et al. 2009; Gibbs and Mackessy 2009) and trait-matching between venom function and broad patterns of snake diet (ibid.). Studies that assess the functional significance of venom variation at the population-level are rare (but see Holding et al. 2016), but are needed to evaluate whether widespread intraspecific variation in venom has functional significance, supporting the idea that variation has evolved because of natural selection rather than neutral evolutionary processes. In particular, studies that use more rigorous hypothesis testing are needed to clarify this matter. By testing for local adaptation in a rattlesnake-prey system, we hope to contribute hypothesis-driven data that informs the venom community's unresolved debate (Sasa 1999; Wüster et al. 1999; Mebs 2001) over whether venom variation in snakes has functional significance in the prey, supporting the adaptive hypothesis of venom variation.

Here we adopt and use data on the toxicity of pigmy rattlesnake (*Sistrurus miliarius*) venom to squirrel treefrog (*Hyla squirella*) prey from different populations to assess whether a local versus foreign pattern of local adaptation is present when specific populations of this system are compared. Venom variation is high at the population-level for this snake (Smiley-Walters, unpublished data). Our goal is to determine if this variation has functional significance in terms of effects on a co-occurring prey species. We used reciprocal experiments in a common garden (laboratory) setting to examine the toxicity of pigmy rattlesnake venom from two study sites in Florida to squirrel treefrogs from the same two locations. Based on the prevalence of venom variation in pit viper systems (Chippaux et al. 1991), the presence of strong positive selection on venom genes of *Sistrurus* rattlesnakes (Gibbs and Rossiter 2008), and the documentation of snake advantage in other rattlesnake-prey systems (Holding et al. 2016), we predict that if a pattern of

local adaptation is documented, measures of venom fitness will indicate an advantage of the pigmy rattlesnake predator rather than a fitness advantage in the prey.

Materials and methods

Study system

Pigmy rattlesnakes are generalist predators whose diet includes mice, lizards, frogs, and centipedes (Gibbs and Mackessy 2009). We chose to work with frogs as a model prey item because anurans comprise greater than 25% of the dietary items found in the gut of pigmy rattlesnakes, a dietary percentage that is comparable only to lizards (Gibbs and Mackessy 2009). Chemical cues from frogs influence the foraging site selection of *S. miliarius* (Roth et al. 1999, Bevelander et al. 2006), suggesting that they are an important prey item. In contrast, snakes do not respond to cues from mice and lizards in selecting microhabitats (Bevelander et al. 2006). We choose to work with squirrel treefrogs because of the ease of collecting this species from our two focal study sites. Additionally, *Hyla sp.* have been documented in the diet of the pigmy rattlesnake (Ernst and Ernst 2011). The pigmy rattlesnake and the squirrel treefrog have large areas of co-occurrence in the southeastern United States (Conant and Collins 1998) and quite likely a long shared evolutionary history.

Collection of study animals and venoms

We used field-collected animals from two locations separated by a Euclidean distance of approximately 340 km: Lake Woodruff National Wildlife Refuge (WOOD) in central Florida (DeLeon Springs, FL) (29.10°, −81.37°) and the Apalachicola National Forest (ANF) in the Florida panhandle (west of Crawfordville, FL) (30.17°, −84.65°). In 2014 and 2015, we collected squirrel treefrogs (*Hyla squirella*) ($n = 151$) at both study sites by hand and using passive PVC pipe traps hung on trees (Boughton et al. 2000). Frogs were transported back to Stetson University where they were held in individual plastic containers and maintained on a diet of crickets until used in toxicity assays. We located pigmy rattlesnakes (*Sistrurus miliarius*) at both study locations by visual survey. Walking searches were conducted at WOOD, while surveys at ANF were mostly conducted by driving, resulting in an increased distance between capture sites in the ANF. We transported snakes back to the lab where they were processed and subsequently returned to their site of capture. We recorded each animal's weight and length and collected venom from each snake by encouraging it to bite a parafilm-covered beaker. Venoms were collected between 2011 and 2013 from WOOD snakes and between 2012 and 2014 from ANF snakes. Venoms were stored at -80° C until use.

For each of the two study sites, we combined individual venom samples collected from that location into a single, pooled stock solution. Pooled venom has been used in previous studies of venom function (Barlow et al. 2009; Richards et al. 2012). We used pooled venom samples here because we were most interested in the population-level response of frogs to venom; we wanted to examine the average toxicity of venom in each population rather than individual venom toxicity. The WOOD pooled venom was obtained from 49 snakes and the ANF pooled venom from 25 individuals. WOOD snakes used in this study were slightly larger [snout vent length (SVL), mean 42.3 cm, range 32.55–54.05 cm] than ANF snakes (SVL mean 34.3 cm, range 26.25–46.05 cm), reflecting the paucity of large snakes observed in the ANF population. All snakes used were greater than 28 cm SVL or 20 g body-weight; these measurements corresponded to pigmy rattlesnakes greater than 1 year in age in a long-term study at Lake Woodruff National Wildlife Refuge (May and Farrell 2012).

Laboratory toxicity assays

Rattlesnake venom contains high concentrations of proteins (~225–250 mg/mL) (Mackessy 2008). We diluted pooled venom from both ANF and WOOD using physiological saline (Scholar Chemistry). Next, we estimated the protein concentration of diluted venoms from absorbance readings at 595 nm using the Bio-Rad Protein Assay Kit (Bio-Rad) and the bovine gamma globulin standard.

Following protein quantification, we weighed squirrel treefrogs that were to be used in the upcoming round of venom injections. Frogs were injected over several months, but each round of injections was paired with respect to venom treatment. Most rounds consisted of 6 or twelve frogs (range 4–19) injected in 1 day. We assigned venom treatments (ANF or WOOD) randomly after stratifying the frogs by weight so that each venom treatment included both large and small animals. Based on the protein concentrations of each solution, we calculated the appropriate volume of diluted venom to deliver to each treefrog to reach a desired body-weight adjusted dose. Additional physiological saline was added to bring the injection volume to 20 µl for each frog. Injections were administered intraperitoneally into the frog's posterior abdominal region. Nine different concentrations (sample size indicated) were used for ANF frogs: 4 (6), 6 (6), 7 (12), 8 (12), 9 (12), 10 (18), 12 (12), 18 (12), and 30 (6) mg/kg. Seven concentrations were used for WOOD frogs: 4 (6), 6 (6), 7 (6), 8 (12), 9 (12), 10 (12), and 18 (1) mg/kg. The concentrations used encompassed the entire dose–response curve (0–100%

mortality). Fewer venom doses were used for WOOD frogs because we reached full mortality at concentrations less than 30 mg/kg and did not have as many frogs from this location. We monitored frogs for mortality at 1, 2, 3, 4, 5, 6, 8, 10, 24, and 48 h following injection. In total, 48 ANF frogs received ANF venom, 48 ANF frogs received WOOD venom, 27 WOOD frogs received ANF venom, and 28 WOOD frogs received WOOD venom.

Data analysis

We used regression techniques to test for interactions between frog origin and venom origin on frog mortality-related data. A significant frog origin by venom origin interaction term would match the local versus foreign definition of local adaptation as described by Kawecki and Ebert (2004). We analyzed data using R version 3.3.1 (R Core Team 2016). First, we fit the entire dataset with a probit regression model that included dose as an explanatory variable and the mortality status at 24 h (dead or alive) as a response variable. Fitting a regression model allowed us to determine the median lethal dose (LD_{50}) for the entire dataset. We used the probit model here because of its long history in toxicity analyses (Finney 1952). We then repeated this process with subsets of data defined by the four unique combinations of frog origin and venom origin. In R, we used the glm function for our probit models. We then used the dose.p function available in the MASS package to estimate the LD_{50} and its associated standard error. Next, we calculated a 95% confidence interval for each of the median lethal dose estimates using the estimated standard error (provided by dose.p) multiplied by the sample size dependent 97.5 percent quantile of the student's t-distribution (using the inverse cumulative probability distribution function).

After computing LD_{50} values, we analyzed the entire data set using a logistic regression model to determine which variables (dose, frog origin, venom origin, and their 2° interactions) were significant contributors to observed frog mortality. We used a logistic regression (glm function in R) for this analysis because of a slightly better fit of this model (lower AIC) compared to the probit model as well as the greater use of logistic regression for significance testing. This model allowed us to test for local adaptation explicitly by examining whether the frog origin by venom origin interaction was significant in explaining frog mortality at 24 h. We used the anova function in R to perform likelihood ratio tests, comparing complex models (containing the variable of interest) and simple models (lacking the variable of interest), to assign a *p* value to each variable.

Lastly, we examined whether the variables in our study (dose, frog origin, venom origin, and their 2° interactions) significantly contributed to the time to death response in squirrel treefrogs. Frog mortality was monitored from 1 to 48 h as described previously. Because frogs could have died anytime between subsequent checks, we defined time to death as the midpoint number of hours between the last check the frog was recorded as alive and the first check it was recorded as dead. We increased the normality of our response variable with a square-root transformation. We then performed a multiple linear regression on the square-root transformed time to death, using the glm function in R, followed by likelihood ratio tests to determine *p* values associated with each variable. In a similar manner to the mortality data, these methods allowed us to test for local adaptation explicitly by examining whether the frog origin by venom origin interaction was significant in explaining time to death in squirrel treefrogs.

Results

In the toxicity trials, 82 of the 151 squirrel treefrogs (54.3%) died during the 24-h interval following venom injection. We estimated the overall LD_{50} for *Sistrurus miliarius* venom on *Hyla squirella* to be 8.68 mg/kg (95% CI 7.56–9.81 mg/kg) using a probit regression model (Fig. 1). The probit-based LD_{50} estimates for each of the four frog origin-venom origin combinations were similar and 95% confidence intervals overlapped substantially (Fig. 2). ANF venom injected into ANF frogs had an LD_{50} of 8.80 mg/kg and ANF venom in WOOD frogs resulted in an LD_{50} of 7.07 mg/kg. WOOD venom

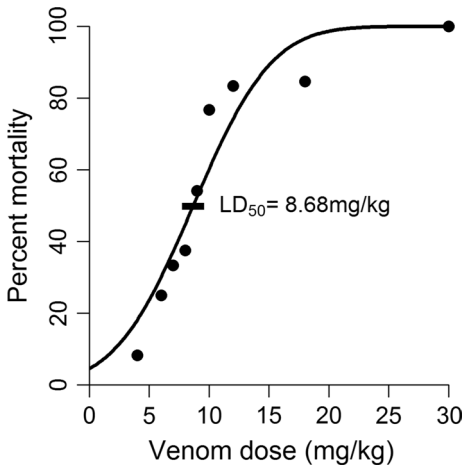

Fig. 1 A probit regression model generated a dose–response curve for pigmy rattlesnake (*Sistrurus miliarius*) venom on squirrel treefrogs (*Hyla squirella*). This *graph* shows the probit regression fitted values (*solid line*) and the summarized mortality data (*solid points*) for all treefrogs ($n = 151$) and both venom sources

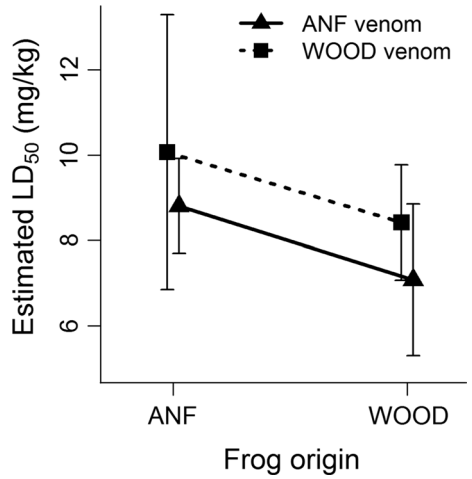

Fig. 2 Median lethal dose (LD_{50}) estimates generated from probit regression models for the four combinations of *Hyla squirella* frog origin (ANF or WOOD) and *Sistrurus miliarius* venom origin (ANF or WOOD). Lower LD_{50} estimates correspond to more toxic venom. Error bars represent 95% confidence intervals

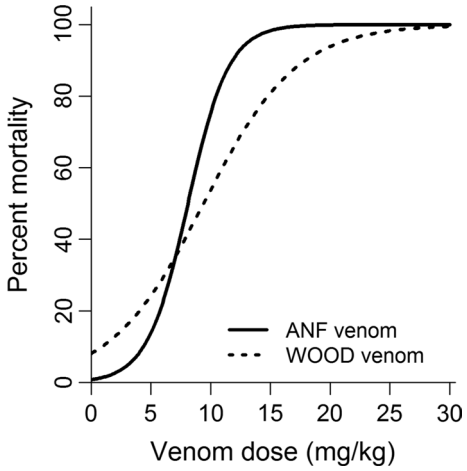

Fig. 3 Fitted values from two logistic regression models of *Hyla squirella* frog mortality over the range of *Sistrurus miliarius* venom doses used in our study. The regression shown by the solid line represents frogs injected with ANF venom, while the regression depicted by the dashed line represents frogs injected with WOOD venom

injected into WOOD frogs had an LD_{50} of 8.42 mg/kg while WOOD venom in ANF frogs resulted in an LD_{50} of 10.07 mg/kg. The confidence intervals associated with LD_{50} estimates, influenced by estimated standard error and sample size, were largest in mismatched pairs (ANF-WOOD) and smallest when the venom source matched the frog source (WOOD venom with WOOD frogs and ANF venom with ANF frogs) (Fig. 2). The relationships between dosage and mortality in most of our datasets were monotonically increasing, the exception being WOOD venom on ANF frogs, but all displayed a constant percent mortality between at least two treatment doses which contributed to larger confidence intervals.

We first analyzed the treefrog mortality response and, as expected, venom dose ($p < 0.001$) was a significant contributor to treefrog mortality at 24 h after injection using a logistic regression. Venom origin ($p = 0.20$) and frog origin ($p = 0.13$) were not statistically significant predictors of frog mortality in the logistic regression model. There was a strong trend towards the venoms from the two populations behaving differently across the range of doses tested; the interaction between dose and venom origin was just outside statistical significance ($p = 0.06$) in our logistic regression. This interaction effect can be visualized by differential shapes of the dose–response curves of the two venoms (Fig. 3); frogs that received the ANF venom treatment resulted in a fitted logistic regression model with a steeper slope than those that received the WOOD venom treatment.

The venom origin by frog origin interaction term was not significant ($p = 0.37$) in explaining frog mortality at 24 h in our logistic regression. Additional secondary interaction terms were also not significant. The lack of a significant interaction between venom origin and frog origin in the treefrog mortality data agrees with the pattern shown by the LD_{50} estimates (Fig. 2). In general, ANF venom was more toxic to treefrogs (ANF venoms displayed lower LD_{50} values) compared to WOOD venom and frogs from ANF were slightly less vulnerable to snake venom (ANF frogs displayed higher LD_{50} values) than frogs from WOOD (Fig. 2). The parallel-line pattern displayed by our LD_{50} estimates (Fig. 2) and the lack of a significant venom origin by frog origin interaction term in our logistic regression analysis are not consistent with the patterns expected under the prediction of local adaptation.

We analyzed the time to death response of treefrogs ($n = 84$) that died within 48 h after venom injection. The square-root transformation of time to death resulted in a better fitting model (AIC = 232) when compared to a model performed on non-transformed data (AIC = 550); the same variables remained significant in both models. Using transformed data, we found that venom dose ($p < 0.001$) and the interaction between frog origin and venom origin ($p = 0.03$) (Fig. 4) were both significant predictors of the time to death for squirrel treefrogs. Venom origin ($p = 0.23$), frog origin ($p = 0.35$), the dose by venom origin interaction ($p = 0.36$), and frog origin by dose interaction ($p = 0.89$) were not significant predictors of a frog's time to death.

The significant interaction between frog origin and venom origin represents a direct test for the presence of local adaptation. This interaction term was significant, displaying a classical crossing pattern when time to death values for sympatric versus allopatric populations were compared (Fig. 4). The average time to death was shorter for

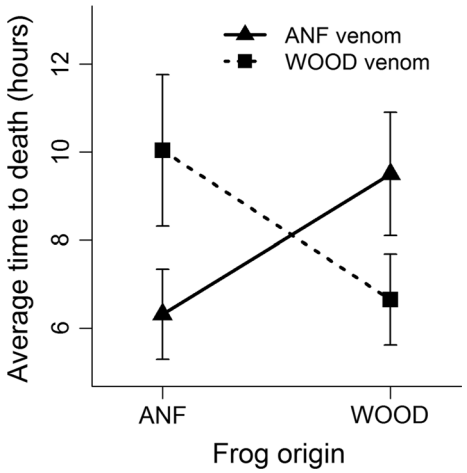

Fig. 4 The average time to death (±1.0 SEM) is shown for the four combinations of *Hyla squirella* frog origin (ANF or WOOD) and *Sistrurus miliarius* venom origin (ANF or WOOD). Shorter times to death favor the snake predator; both venoms caused shorter times to death in sympatric frogs and longer time to death in allopatric frogs

sympatric pairs: ANF frogs injected with ANF venom had a mean time to death of 6.32 h ($n = 28$) and WOOD frogs injected with WOOD venom had a mean time to death of 6.65 h ($n = 13$). In contrast, the average time to death was longer for mismatched combinations: ANF frogs injected with WOOD venom had a mean time to death of 10.04 h ($n = 27$) and WOOD frogs injected with ANF venom had a mean time to death of 9.50 h ($n = 16$). The fitness trade-off pattern displayed by our time to death data (Fig. 4) and a significant venom origin by frog origin interaction term for this response variable in our multiple regression analysis support the presence of local adaptation in this measure of venom function.

Discussion

Measures of prey mortality

A strength of this study is that it used a direct measure of prey mortality and, therefore, is based on a measure of venom function that is closely tied to snake fitness. Our findings complement recent work by Holding et al. (2016) in which indirect measures of venom effectiveness (inhibition of venom enzymatic activity) and an explicit statistical approach were used to test for local adaptation. Our ability to detect local adaptation in the *S. miliarius* and *H. squirella* predator–prey system, however, differed depending on the measure of prey mortality that was used. Specifically, we found evidence of local adaptation using the response of time to death of the squirrel treefrog prey, but not using a more traditional measure, frog mortality at 24 h (LD_{50}). This raises the issue of which measure of venom toxicity is more ecologically and evolutionarily relevant for assessing local adaptation in natural populations.

In terms of biological relevance, the LD_{50} and the time from envenomation-to-death likely differ in how well they measure whether an envenomated prey represents a potential meal for a snake predator. As other authors have noted (Chiszar et al. 1999; Barlow et al. 2009), the LD_{50} of venom has limited ecological relevance because prey that die near the commonly used cut-off times of 24 or 48 h are unlikely to result in a meal for a snake that engages in strike and release predation. This is because if the prey takes a prolonged time to die then the snake may be unable to re-locate the prey post-strike during a longer envenomation-to-death time frame. In contrast, prey that die or are immobilized quickly represent prey that are more likely to be consumed.

Another issue pertinent to the ecological relevance of our results is that we conducted toxicity tests with diluted venom. As venom concentration increases towards volumes realistically delivered by the snake to the prey, all prey that receive an accurate strike will likely die from the encounter, while time to death may scale in a more direct manner with dosage. In other words, venom that causes more rapid mortality at lower doses may also kill comparatively faster at higher doses, but venom that fails to kill at low doses may be as effective as more toxic venom once the concentration is increased. As dose increases, time to death may occur at shorter time-scales. Mackessy (1988) observed a decrease in the time to incapacitation and time to death of lizards with increased dosage of *Crotalus viridis* venom. In our study, we saw decreased times to death at higher doses, but the specific relationship of this scaling beyond the doses tested is unknown. It is also possible that variability in strike accuracy is an important determinant of whether local adaptation in venom function becomes biologically relevant in an interaction between predator and prey. Specifically, Kardong (1986) found that imperfect strikes resulted a prolonged time to death, seven times longer than unflawed "normal" strikes. Imperfect snake strikes may occur more often than we currently recognize. Overall, we believe that time to death is a more ecologically relevant measure of venom function than the binomial mortality response associated with LD_{50} and so it is a more appropriate measure of venom effectiveness when assessing local adaptation in this rattlesnake-prey system.

Despite the above issues, median lethal dose (LD_{50}) is a commonly reported measure of function in studies of snake venom toxicity (D'Império Lima et al. 1991; da Silva and Aird 2001; Mebs 2001; Mackessy et al. 2006; Mackessy 2008; Barlow et al. 2009; Gibbs & Mackessy 2009; Mackessy 2010; Richards et al. 2012; Bénard-Valle et al. 2014; Lomonte et al. 2014; Laustsen et al. 2015) whereas

studies that collect both whole-organism response data and the measure of LD_{50} are rare (but see Mackessy 1988; Barlow et al. 2009; Richards et al. 2012). As discussed above, the qualities that make the median lethal dose good for studies of comparative toxicity do not necessarily make it the best metric for evolutionary studies of venom. An LD_{50} is a summary statistic, generating a single measure of mortality from several individual data points. Each mortality response is binomial, having two possible outcomes (dead or alive). Other response variables, such as time to death, can have many possible outcomes making it easier to detect small differences and provide a more sensitive assessment of venom function. Our results suggest that researchers conducting evolutionary studies of venom variation should assess venom effectiveness not only with the measure of LD_{50}, but with other measures of venom function, such as time to death, that capture additional dimensions of the way venom acts on prey.

Population specific effects of venom

Our finding that sympatric venom-prey pairs resulted in more rapid mortality of treefrog prey compared to allopatric combinations of venom and prey is consistent with an antagonistic interaction where the snake predator is locally adapted and evolutionarily ahead of the prey with which it interacts. Our finding that the snake is ahead of the prey is counter to that proposed under the life-dinner principle, where selection is predicted to be stronger on prey compared to predators, favoring prey advantage in an arms race (Dawkins and Krebs 1979). Our finding is in agreement with snakes being evolutionarily ahead of their prey, as reported by Holding et al. (2016) in another rattlesnake-prey system. As discussed by Holding et al. (2016), possible reasons for the snake predator displaying a pattern of local adaptation (and conversely the prey being locally maladapted) are: (1) a greater fitness consequence for the snake, relative to the prey, in this predator–prey interaction or (2) it is easier for the snake to evolutionarily modify venom proteins than it is for the prey to modify target molecules that may be biologically conserved for other functions. Future directions for this work include: (1) expanding our analyses beyond two populations to include tests of predator and prey populations at a range of spatial scales (Hanifin et al. 2008), (2) using proteomic techniques that isolate venom components to determine the specific venom protein(s) [or synergistic combinations of proteins (Borkow et al. 1993)] that may be responsible for the differences in time to death that we observed (Modahl et al. 2016), and (3) examining if local adaptation of the pigmy rattlesnake to more quickly kill squirrel treefrogs limits its ability to be locally adapted to other prey species that also makeup its diet.

In biological systems, the likelihood of local adaptation is influenced by a number of factors including the relative rate of gene flow in the two interacting species (Hoeksema and Forde 2008), the size of a population through the effect on the amount of standing genetic variation (Leimu and Fischer 2008), and the degree of specificity of the antagonistic relationship in parasite–prey systems (Lajeunesse and Forbes 2002). The amount of habitat divergence (Hereford and Winn 2008), but not geographical scale of a study (Leimu and Fischer 2008) has also been shown to influence the ability to detect local adaptation in the field. In our system, selection is likely diffuse because of the broad diet of the pigmy rattlesnake (Gibbs and Mackessy 2009) and multiple predators that feed and, hence exert selection on squirrel treefrogs (Binckley and Resetarits 2002; Smith 2005; Toledo et al. 2006). Population sizes of both pigmy rattlesnake predator and treefrog prey are likely large (May et al. 1996; Farrell et al. 2011; authors' personal observation), suggesting stochastic effects such as genetic drift (Kawecki and Ebert 2004) in driving the evolutionary change of either snakes or prey in these populations are limited.

Finally, in addition to documenting the presence of snake local adaptation in this system, we also show that venoms from different populations of snakes have different actions over time on treefrog prey. Specifically, we found a venom origin by dose interaction in our frog mortality data, showing that ANF venom generated a steeper dose–response curve when compared to venom from the WOOD population of snakes. Synergistic effects between different venom proteins could generate this type of a functional venom difference (Borkow et al. 1993). Also present in our data was a weak pattern showing ANF venom outperforming WOOD venom with regards to the LD_{50} in both populations. If venom function is related to diet (Barlow et al. 2009), this trend may indicate a greater reliance on small treefrog prey in the diet of ANF snakes compared to WOOD snakes. Lastly, our time to death data indicate that venom origin is an important factor in explaining how quickly a prey item dies. Combined with our detection of local adaptation of venom in this system, we conclude that not only are snake venoms from different populations functioning differently, but they may also act in a way that enhances the snakes' foraging success on treefrogs in this system. Overall our findings support the hypothesis of Daltry et al. (1996) that the widespread presence of population-level variation in snake venom is adaptive.

Acknowledgements We thank Hannah VanSumeren for her assistance with lab work. S. Mackessy provided helpful advice for the development of LD_{50} assay protocols. We also thank numerous students, visiting researchers, and collaborators for help locating snakes in the field including C. Lind, J. Serrao, M. Pilgrim, E. Royal, D. Rokyta, M. Margres, K. Wray, M. Holding, R. Denton, and D. Salazar-Valenzuela. Our manuscript was improved, thanks to comments

from G. Zancolli and one anonymous reviewer. Venoms from pigmy rattlesnakes were collected under a License to Possess or Exhibit Venomous Reptiles and/or Reptiles of Concern from the Florida Fish and Wildlife Conservation Commission with the following license renewal identifications (year): 411-75193 (2011–2012), 411-95180 (2012–2013), 411-104419 (2013–2014), and 411-115575 (2014–2015). Squirrel treefrogs were obtained under a Scientific Collecting Permit from the Florida Fish and Wildlife Conservation Commission (permit number LSSC-11-00067A).

Author contribution statement SASW and HLG conceived the study. SASW and TMF collected organisms in the field and performed statistical analyses. SASW conducted laboratory experiments and wrote the manuscript. All authors were involved in manuscript revisions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed. Research methods were approved by the Stetson University IACUC (protocol #2011TF101).

Funding This research was made possible by financial support from The Brown Visiting Teacher-Scholar Fellows Program at Stetson University.

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